SEQUENCING METHOD

Introduction

Determination of the sequence of nucleotide bases in 2'-deoxyribonucleic acid (DNA) and to a lesser extent ribonucleic acid (RNA) is a central technology for much of present day molecular biology and its many applications. There are currently two main methods available for sequencing DNA, and both involve using a DNA polymerase enzyme to make a copy of a suitably primed strand of the unknown DNA which acts as a template. In the so-called chain-termination method of Sanger et al (1977), the growing copy strand is randomly (at least, randomness is intended) terminated by incorporation of a 2', 3'-dideoxyribonucleotide (ddNTP) competing with the normal 2'-deoxyribonucleotide (dNTP) for addition to the 3'-end of the growing copy strand. By using suitable conditions and four separate preparations corresponding to dATP with ddATP, dCTP with ddCTP, dGTP with ddGTP and dTTP with ddTTP, all possible lengths of terminated copy strands are available. They are separated by size by gel electrophoresis, and detected by means of an incorporated label, commonly radioactive but more recently fluorescent (Smith et al, 1986; Ansorge et al, 1987). The DNA sequence of the copy, and therefore the complementary sequence of the template, is derived from the pattern of chain lengths detected for the separate A, C, G and T termination mixture.

The chemical degradation method of Maxam and Gilbert (1977) also uses DNA polymerase catalysed formation of a template-copy. The dNTP's are radioactive with ^{32}P (or ^{35}S) at the α-position, so the copy is also radioactive. Chemical treatments specific for

different bases cleave the copy into sets of fragments which, as with the Sanger dideoxy chain termination method, can be separated by gel electrophoresis and detected (by autoradiography) to yield patterns from which the DNA sequence can be deduced. Variants of these methods have been described (Church & Kieffer-Higgins, 1988; Gish and Eckstein, 1988), but are still dependent upon gel electrophoresis to separate DNA fragments, and upon such methods such as radiolabelling for detection of the separated fragments.

All of these methods are slow, complex and expensive in relation to the needs for DNA sequencing. For example, the best rates of DNA sequencing to date are about 600 nucleotides in 12 hours, using an automated instrument (Landegran et al, 1988). This corresponds to 50 bases per hour or 0.014 bases per sec. This rate is approximately two-orders of magnitude lower than that thought necessary for large-scale sequencing in the Human Genome Project (Alberts et al, 1988).

Three laboratories have reported attempts to avoid the needs for copy-strand fragments and their length-determination by gel electrophoresis. Hyman (1988) immobilised the DNA template primer with polymerase on an ion-exchange material and exposed the complex to a flow wherein only one of the four dNTP's was present at a time. A downstream detection system then detected the pyrophosphate liberated in the DNA polymerase reaction:-

template - primer + dNTP -> template - (primer + dNMP) + PPi

where dNMP stands for the added nucleotide residue, PPi for pyrophosphate. The sequencing rate was approximately 1 base every 10 minutes. Melamede (1987) also proposed the use of an immobilised DNA template, primer and polymerase complex exposed to a flow containing only one species of dNTP at a time.

However, incorporation of dNTP into the copy was calculated not from measurements of pyrophosphate release but from the difference in dNTP concentrations entering and leaving the flow cell which contains the complex of DNA template and polymerase. No experimental data were presented.

Jett (1989) provided a single stranded DNA or RNA sequence of labelled nucleotides, complementary to the sequence to be determined, suspended in a moving flow stream. An exonuclease sequentially cleaved individual basis from the end of the suspended sequence. The resulting train of individual labelled nucleotides was passed to a downstream location for analysis of the individual nucleotides.

This invention

As with the methods of Melamede (1987) and Hyman (1988), we also use an immobilised complex of template and primer, exposed to a flow containing only one dNTP at a time. We differ however in that we directly measure the growth of the template copy, rather than infer it indirectly from changes in the composition of the flow medium. The methods of detection are preferably spectroscopic and do not require the further addition of chemical reagents to effect measurement of template-copy growth. The spectroscopic methods are:-

i) Evanescent wave spectroscopy
ii) Fluorescence detection (non-evanescent wave)
iii) Absorption spectroscopy (non-evanescent wave)

Alternatively the individual nucleotides may be labelled, e.g. radioactively. Attachment of each successive labelled nucleotide to the immobilised complex is then detected by an increase in total radioactivity.

Irrespective of which detection method is used, the time-dependent signal arising from

polymerase catalysed template copy growth depends on the availability of the correct (i.e. complementary) nucleotide for base-pairing with the template at the growing point of the copy. If all four nucleotides are absent, there is no polymerase activity. If only one nucleotide is present, then extension of the copy will occur only as far as base-pairing with the template is possible in the absence of the other three nucleotides.

Preferably a continuous flow of pulses of deoxynucleotides (dATP, dCTP, dGTP, dTTP) or ribonucleotides (ATP, CTP, GTP, UTP)or analogues of these nucleotides, each separated by a wash pulse, is passed over the tethered template DNA or RNA (with any extension and linkers, and the primer oligonucleotide). This is done under conditions, e.g. in the presence of a suitable polymerase enzyme, to cause an extension of the primer by addition of the nucleotide (or analogue) to the 3'-end of the primer if the nucleotide (or analogue) is complementary to and can hybridize with the next free nucleotide of the template (going from the 3'-end towards the 5'-end). Conditions of solvent, temperatures, ionic strength and concentrations of any polymerase enzyme activating cations and anions are maintained such that the polymerase is enzymatically active in the presence of its substrates. Because only one of the four nucleotides is present at a time, extension of the copy strand occurs in jumps depending on whether the appropriate nucleotide for pairing with the next unpaired base of the template strand is present or not. Each such jump is herein designated a polymerisation event. Thus the signal also exhibits time-dependent jumps that reflect the extension jumps (polymerisation events) of the copy strand. Furthermore, the size of the signal jumps is proportional to the number of bases added in each

extension jump (i.e. proportional to the number of polymerisation events), e.g. the addition of say three adenine residues gives three times the signal given by the addition of only one such residue. Because the copy strand can be extended by only an integral number of bases (0, 1, 2, 3...) during any extension jump, then the optical signals are quantised. This property of the signals is highly advantageous for calibration and for discrimination against noise.

For simplicity, this invention is hereafter described in terms of DNA sequencing, using a DNA template with DNA polymerase and deoxynucleotides (dNTP's) or analogues to make a DNA copy. It should be noted that RNA technologies could be used : e.g. (i) DNA as the template, RNA polymerase to make an RNA copy (ii) RNA as the template, reverse transcriptase to make a DNA copy, and (iii) RNA as the template, RNA replicase to make an RNA copy.

(i) Evanescent wave spectroscopy (EWS)

Evanescent wave spectroscopy is defined in the present context as embracing three related methods: (i) attenuated total reflection (ATR) spectroscopy (ii) total internal reflectance fluorescence (TIRF) spectroscopy, and (iii) surface plasmon resonance (SPR) spectroscopy. Each of these three spectroscopic techniques examines an optical property of a solution bordering a surface where total internal reflection of a light beam has occurred. In each case the incident and reflected beams are on the side of the surface distal to (i.e. remote from) the solution under study, whereas the evanescent wave is established on the solution side of the surface but extends into that solution for a very short distance, typically less than the wavelength of the incident/reflected beam. Spectroscopy by ATR or TIRF requires only a transparent material such as glass or quartz to create the

interfacial surface with the solution, whereas SPR spectroscopy requires that the glass or quartz surface be coated with a thin (e.g. 50nm) metal layer of, for example, silver. All three methods can detect the exchange of solute molecules between the bulk phase of the solution and the interfacial surface, albeit by different means. ATR spectroscopy detects the absorption of evanescent wave light by molecules, with appropriate absorption spectra, that lie within the evanescent wave region. If the absorbed light is re-emitted as fluorescence then the emission can be measured with a suitable detector, such as a photo-multiplier tube, leading to TIRF spectroscopy. Thus both ATR and TIRF spectroscopy measure the absorption of light by molecules at or close to the interfacial surface, the difference being that ATR measures the absorption directly whereas TIRF measures it indirectly, as re-emitted fluorescence. By contrast, SPR spectroscopy measures changes of refractive index that may occur in the SPR evanescent wave region, but, just as with ATP and TIRF, those changes arise due to redistribution of molecules between the bulk phase of the solution and the evanescent wave region. For a review of these methods, see Sutherland and Dahne, 1987.

Having given a brief outline of evanescent wave spectroscopies, we can describe their application to DNA sequencing. The DNA molecule of unknown sequence, derived from biological techniques such as DNA cloning or polymerase chain reactions must (i) be single stranded (ii) be attached either directly or indirectly by either its 3' or 5' end to the interfacial surface of an EWS device, and (iii) possess a known sequence, either naturally occurring or added, at its 3' end such that a complementary oligonucleotide (primer) can be hybridised to create a run of double

stranded DNA. Thus in the presence of DNA polymerase and deoxyribonucleotide triphosphates (dNTP) the unknown DNA acts as a template for the synthesis of a complementary copy extending by growth from the 3' end of the primer. DNA polymerase catalysed growth of the template copy results in recruitment of dNTP's from the bulk phase to the interfacial region where the template is tethered. It is assumed that the copy strand remains base-paired to the template strand, and does not diffuse away. Template copy growth can therefore be measured by EWS spectroscopy. ATR spectroscopy requires that the incident beam be in the wavelength region of a major absorption band of the dNTP's, typically about 260nm. TIRF spectroscopy requires that the nucleosides incorporated into the template copy can be excited to fluoresce by the evanescent wave : fluorescent DNTP analogues are required. SPR spectroscopy has fewer limitations : dNTP analogues are not required (although substitution with refractive index enhancing atoms such as Br, I or Hg might be advantageous for sensitivity) and the choice of wavelength is not critical.

Figure 1 illustrates the experimental set up, shown for the specific case of SPR spectroscopy. The 3' end of the template DNA (or its extension) is attached to the interfacial silver surface, and the primer oligonucleotide is hybridised to the template. Alternatively the primer could be attached to the surface and the template DNA hybridised to the primer. Clearly the polarity of attachment can be reversed:- the 5' end of the template bound to the surface, and the primer hybridised to the free 3' end of the template or extension.

Figure 2 shows idealised experimental results using SPR spectroscopy. The sequence of the template being copied is read directly from the associations

between jumps in the EWS signal and the particular dNTP pulse present at the template site.

(ii) Fluorescence detection

Because each cycle of dNTP (or rather its fluorescent analogue) is preceded and followed by a wash, EWS (TIRF) is not mandatory to distinguish the fluorescence signal arising from the template-copy from free nucleotides in the medium. Thus a more conventional optical geometry can be used, and immobilisation of the template-primer-polymerase complex can be on beads, fibres or membranes rather than an evanescent wave surface.

A serious drawback of the fluorescent method for DNA sequencing, irrespective of whether or not it uses an evanescent wave, is the fact that the absolute size of the signal grows as the copy strand grows. This makes it harder to detect the increments (jumps) of fluorescence resulting from strand extension.

Even under ideal conditions (Poissonian photon-counting statistics, zero background and noise-less excitation source), it is necessary to increase the measurement time for each increment of nucleotide addition in proportion to the number of such increments in order to maintain constant sensitivity for the detection of each successive fluorescence increment. For example, if it took 1 sec to measure the fluorescence of the first added nucleotide to a satisfactory accuracy, then 10 seconds would be needed to measure the tenth added nucleotide, 100 seconds for the hundredth, and so on. Thus what is intended to be a fast DNA sequencing method becomes progressively slower as it proceeds.

There are two instrumental solutions to this problem. The first is to increase not the duration of measurement for successive increments of fluorescence, but the excitation intensity. By this means the

accumulation of photon counts required for accuracy can be achieved in a constant time. Technically this can be arranged with a CW laser excitation source equipped with an acousto-optic or electro-optic modulator to modify the beam intensity transmitted to the sample. The second solution is to irreversibly photobleach the sample fluorescence with an intense light pulse at periodic intervals, thereby resetting the sample fluorescence to zero before it can significantly degrade the detection of successive increments. Technically this can also be achieved with a CW laser equipped with a modulator (Garland, 1981).

The fluorescent probe itself can be attached to each of the four dNTP's. As each dNTP is presented separately to the template-primer-polymerase complex, the same fluorophoric group can be used for each dNTP. Alternatively, fluorescent dideoxy chain terminators can be used in competition with unlabelled dNTP's, under conditions where the number of terminated chains is not so large as to deplete the number of unterminated chains to the point where fluorescence increments associated with chain termination become difficult to detect. Each dNTP is presented with its corresponding fluorescently-labelled ddNTP to the template-primer-polymerase complex, and a single fluorophore is sufficient.

(iii) Absorption spectroscopy

The direct measurement of template copy growth by detecting the increase in ultraviolet light absorption at approximately 250-260nm caused by addition of new nucleotides to the copy strand is not sensitive. However, the availability of relatively large amounts of starting material (template) through DNA cloning or polymerase chain methods, allied to powerful U-V laser sources such as frequency-tripled Nd-Yag lasers, makes it technically possible to measure

template-copy growth directly by absorption spectroscopy.

The advantages of these optical methods over existing methods are very large. They include:-

- potential for high speed (e.g. 10 bases/sec)
- miniaturisation and parallel flow lanes
- no need for gel electrophoresis
- direct signal transfer to computer
- no need for radioactive nucleotides
- greater chance for complete automation due to absence of gel separation steps.

The following Examples illustrate the invention.

EXAMPLE 1

Detection of Polymerase Catalysed Nucleotide Addition on a Silver Slide using Surface Plasmon Resonance

Materials and Methods

Reagents

1. 0.25pmole/µl solutions of hybrid DNA (consisting of a 17 nucleotide primer sequence hybridised to a 20, 27 or a 97 nucleotide template sequence) prepared in 10 mM phosphate buffer pH 7.4 containing 10mM phosphate buffer pH 7.4 containing 10mM $MgCl_2$ and 50mM NaCl.
2. 10mM phosphate buffer pH 7.4 containing 10mM $MgCl_2$ and 50mM NaCl (sequencing phosphate).
3. 10mM phosphate buffer pH 7.4.
4. DNA Polymerase 1 "Klenow fragment" diluted in sequencing phosphate containing 250µM dATP, dCTP, dGTP and dTTP.
5. Block solution of 0.05% BSA in sequencing phosphate.

Equipment

Rig

A single beam direct reference rig using a 632.8nm wavelength helium neon laser which interrogated two spots and subtracted one signal from the other, was used.

Pump and flow cell

A modified step motor pump which held 2 syringes was used to deliver different or identical solutions to the 2 segregated areas of the flow cell.

Silver Slides

Silver coating thickness on the slides was greater than that normally used for SPR work i.e. 60nm to achieve an SPR width of approximately 0.6 degrees at approximately 5% from the reflectance minimum.

Slide Tolerance Limits

The slides were rejected if the SPR profiles on both areas of the silver slide were not similar in shape and minimum.

Experimental Procedure

1. 1ml 10mM phosphate buffer ph 7.4 was added at 4µl/second to the silver slide.
2. 1ml sequencing phosphate was added at 4µl/second.
3. 1ml of hybrid (17/20 was used as the reference DNA) 17/27 or 17/97 was added at 2µl/second followed by 3 washes in sequencing phosphate.
4. 1ml of BSA block solution was added at 2µl/second followed by 3 x 0.3ml washes in sequencing phosphate.
5. 1ml of Klenow plus nucleotides was added at 2µl/second followed by 3 x 0.3ml washes in sequencing phosphate.

Angle and time scans were obtained at strategic points during the experiment. The overall shift difference caused by the addition of extra nucleotides (3 for the reference, 10 for the 17/27 and 80 for the 17/97) was recorded.

Results

Hybrid	Hybrid coverage moles/mm^2	Nucleotide added moles/mm^2	SPR shift % reflectivity
17/97	3×10^{-15}	2.3×10^{-13}	3
17/27	Not determined	Not determined	2

The figures in the last column are additional changes over and above those of the 17/20 reference.

Conclusion

The SPR signal obtained from the addition of ten nucleotides to a primer-template complex by the Klenow fragment of DNA polymerase could be measured above the signal obtained from a three nucleotide addition in the reference sample. This clearly demonstrates the detection by SPR of the addition of seven nucleotides.

If the following change is made to the equipment then single nucleotide addition will be detected by SPRS.

The experiments were conducted by recording

one reflectivity measurement per second. This gives signal to noise resolution down to seven nucleotides. However by increasing the measurement rate to 1000 per second an improvement by a factor of 30 can be achieved which will permit detection of single nucleotide addition. Noise is proportional to the square root of the number of readings taken, therefore the square root of 1000 equals 30.

EXAMPLE 2

Polymerase Catalysed Single Nucleotide Addition to template DNA Immobilised on Silver Slides

Method

1. Template-primer DNA (a 17 nucleotide primer annealed to a 97 nucleotide template) was immobilised on the silver slides at a coverage of 1.25×10^{-13} moles/silver slide.

2. The 4 nucleotide / polymerase mixes were made up as follows in 10mM phosphate buffer pH 7.4 containing 10mm $MgCl_2$ and 50mm NaCl:-

a) 5µM deoxyadenosine 5'-triphosphate + 200U/ml Klenow fragment (Amersham T2141Z),

b) 5µM deoxycytidine 5'-triphosphate + 200U/ml Klenow fragment,

c) 5µM deoxyguanosine 5'-triphosphate + 200U/ml Klenow fragment,

d) 5µM deoxythymidine 5'-triphosphate + 200U/ml Klenow fragment,

To each mix 100µCi/ml of the corresponding ^{32}P labelled deoxynucleotide triphosphate was added (Amersham PB204, 205, 206 and 207).

The mixtures a-d were added singly in the sequence in which they appeared in the template strand as follows:-

Single nucleotide additions

1. The silver slide was placed on the biosensor hemicylinder and its position marked. The flow cell was placed on top of the slide.
2. 3ml of 10mM phosphate buffer pH 7.4 was flowed over the silver slide.
3. 1ml of 10mM phosphate buffer pH 7.4 containing 10mm $MgCl_2$ and 50mm NaCl was flowed over the silver slide.
4. 100µl of the nucleotide / enzyme mix was injected and left on the silver slide for 5 minutes at room temperature.
5. The silver slide was washed with 5ml of 10mM phosphate buffer pH 7.4 containing 10mm $MgCl_2$ and 50mm NaCl + 0.005% Tween 20.
6. The silver slide was removed and blotted to remove any surface fluid then counted on the tritium channel of a scintillation counter.

Incorporation of the deoxynucleotide was determined from the increase in counts on the silver slides and calculated from the specific activity of the nucleotide/polymerase mixture used at that step.

The results are shown in Table 1.

7. The silver slide and flow cell were replaced on the biosensor in the same position and steps 3-6 were repeated with the next nucleotide/polymerase mixture until the predetermined nucleotide addition sequence was completed.

Control silver slides were subjected to the same nucleotide addition procedure.

Results

Correct Sequence Single Nucleotide Addition to Silver Slide Immobilised DNA

Addition Nos.	Nucleotide Added	Radioactivity (counts per minute)	Moles nucleotide added /slide
1.	T	127	7.5×10^{-15}
2.	G	253	1.3×10^{-14}
3.	T	651	2.3×10^{-14}
4.	G	702	5.3×10^{-15}
5.	A	925	1.8×10^{-14}

Conclusion

The addition of successive single nucleotides by the Klenow fragment of DNA polymerase 1 to this primer template hybrid immobilised at the silver surface can be observed with radioactively labelled nucleotides.

In the absence of the primer template hybrid no successive increase in nucleotide addition is observed.

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CLAIMS

1. A method of sequencing a single-stranded nucleic acid chain, which method comprises:-

a) providing a template-primer complex comprising the single-stranded chain (the template) and at its 3'-end a double stranded chain (comprising the primer), the complex being attached at one end to a solid surface,

b) presenting a single nucleotide or nucleotide analogue to the complex in the presence of a polymerase under conditions to cause an extension of the primer (a polymerisation event) by addition of the nucleotide (or analogue) to the 3'-end of the primer if the nucleotide (or analogue) is complementary to and can hybridize to the next free nucleotide at the 5'-end of the template, and

c) repeating step b) successively using different nucleotides or nucleotide analogues,

d) the method being characterized by detecting a change in the template-extended primer complex resulting from each polymerisation event that occurs during performance of steps b) and c).

2. A method as claimed in claim 1, characterised by detecting, directly and by spectroscopic means, each polymerisation event that occurs during performance of steps b) and c).

3. A method as claimed in claim 2, wherein each polymerisation event is detected by evanescent wave spectroscopy.

4. A method as claimed in claim 2, wherein each polymerisation event is detected by attenuated total reflection spectroscopy.

5. A method as claimed in claim 2, wherein fluorescent nucleotide analogues are used in steps b)

and c) and each polymerisation event is detected by total internal reflectance fluorescence spectroscopy.

6. A method as claimed in claim 2, wherein each polymerisation event is detected by surface plasmon resonance spectroscopy.

7. A method as claimed in claim 6, wherein high refractive index nucleotide analogues are used in steps b) and c).

8. A method as claimed in claim 2, wherein fluorescent nucleotide analogues are used in steps b) and c) and each polymerisation event is detected by fluorescence spectroscopy.

9. A method as claimed in claim 2, wherein each polymerisation event is detected by absorption spectroscopy.

10. A method as claimed in any one of claims 1 to 9, wherein the single-stranded nucleic acid chain is DNA.

11. A method as claimed in any one of claims 1 to 10, wherein a continuous flow of pulses of deoxynucleotides (dATP, dCTP, dGTP, dTTP) or ribonucleotides (ATP, CTP, GTP, UTP) or analogues of these nucleotides, each separated by a wash pulse, is passed over the complex attached to the solid surface.

DNA SEQUENCING BY SPRS DETECTION OF TEMPLATE COPY GROWTH:
EXPERIMENTAL ARRANGEMENT

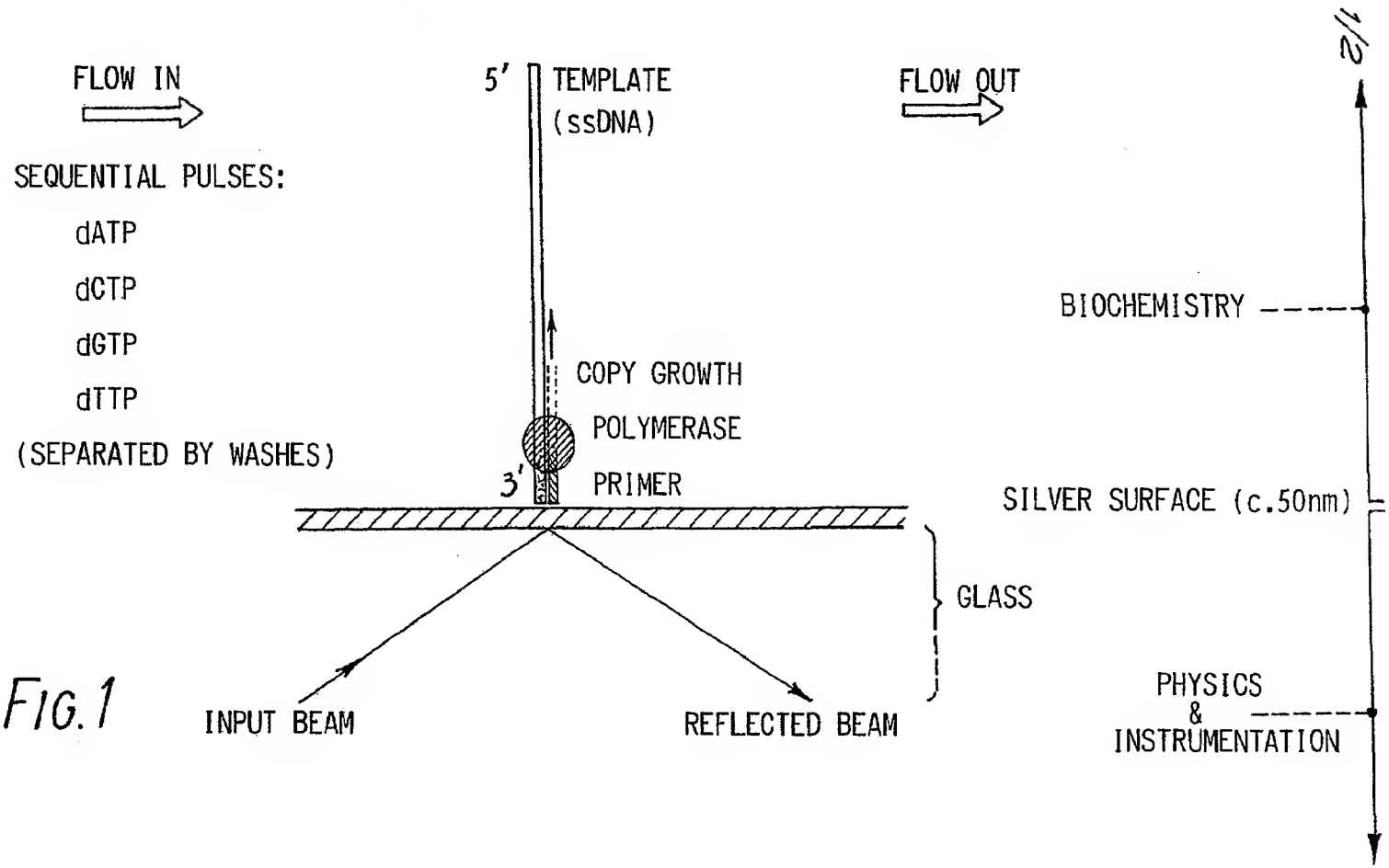

FIG. 1

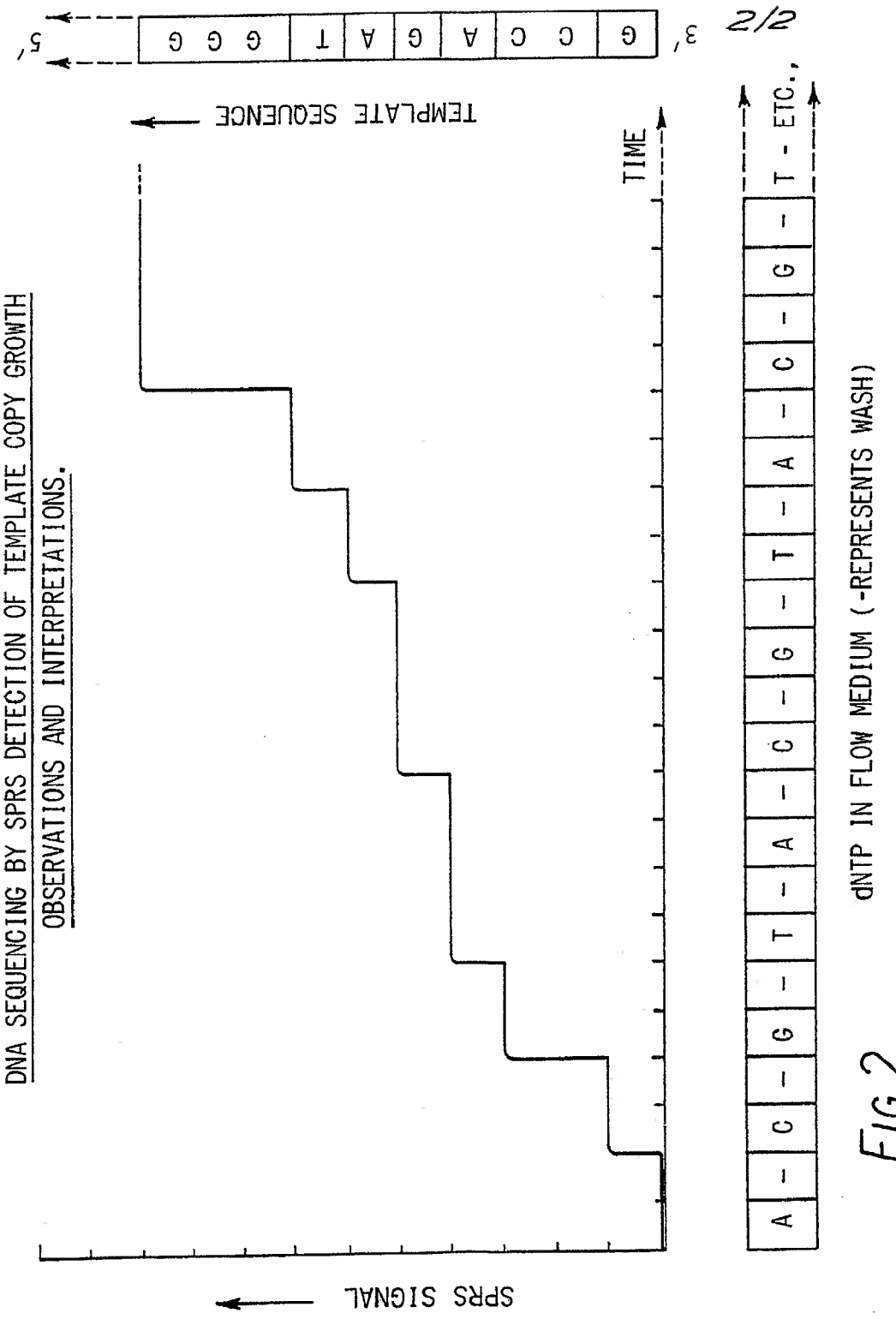
DNA SEQUENCING BY SPRS DETECTION OF TEMPLATE COPY GROWTH
OBSERVATIONS AND INTERPRETATIONS.
5'
G G G T A G A C C G 3'
TEMPLATE SEQUENCE
TIME
SPRS SIGNAL
A - C - G - T - A - C - G - T - A - C - G - T - ETC.,
dNTP IN FLOW MEDIUM (-REPRESENTS WASH)

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/00726

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A, 0223618 (NEW YORK MEDICAL COLLEGE) 27 May 1987 see column 4, lines 28-60; column 6, line 44 - column 7, line 14; column 15, line 34 - column 16, line 15 cited in the application	1-10
Y	US, A, 4770992 (G.J. VAN DEN ENGH et al.) 13 September 1988 see column 7, line 60 - column 8, line 24	1-10
Y	EP, A, 0233053 (APPLIED BIOSYSTEMS) 19 August 1987 see page 2, lines 13-15; page 8, lines 13-30; page 22, line 1 - page 27, line 9	1-11

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th July 1990	03 AUG 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO, A, 89/03432 (UNITED STATES DEPARTMENT OF ENERGY) 20 April 1989 see the whole document cited in the application	1-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. GB 9000726
SA 36812

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 26/07/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0223618	27-05-87	US-A- 4863849	05-09-89
		JP-A- 62085863	20-04-87
US-A- 4770992	13-09-88	None	
EP-A- 0233053	19-08-87	US-A- 4855225	08-08-89
		JP-A- 62249049	30-10-87
WO-A- 8903432	20-04-89	None	

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82